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Purification and Characterization of an Osteoclast Membrane Glycoprotein With Homology to Manganese Superoxide Dismutase

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Abstract The osteoclast is the specialized multinucleated cell primarily responsible for the degradation of the inorganic and organic components of bone matrix. Isolated avian osteoclasts have been used to immunize mice and generate an osteoclast-directed monoclonal antibody library (*J. Cell Biology,* 100:1592). A subset of these monoclonal antibodies recognizes antigens which are expressed on osteoclasts and which are absent or nearly so on multinucleated giant cells formed in vitro from monocyte or marrow mononuclear cells. One of these antibodies, designated 121F, has been used to identify and purify an osteoclast plasma membrane-associated glycoprotein. Western blot analysis on disulfide bond-reduced extracts from osteoclasts or multinucleated giant cells formed in vitro demonstrates that the 121F antibody recognizes a 150 kDa protein detectable only in osteoclasts. This high molecular weight protein has been purified by a combination of immunoaffinity and gel filtration chromatography procedures, in conjunction with electroelution of a single band from SDS-polyacrylamide gels. Silver staining of the purified antigen on SDS-polyacrylamide gels has revealed a single protein species larger than 200 kDa in its unreduced form and 150 kDa when disulfides are reduced. Isoelectric focusing of the purified antigen reveals a single species, having a neutral pI point of 6.95. Whereas endoglycosidase treatment and lectin affinity chromatographic analyses demonstrate that the antigen recognized by the 121F antibody possesses complex N-linked sugars, trifluoromethanesulfonic acid treatment indicates there are no additional O-linked carbohydrate components. Periodate oxidation and monosaccharide hapten inhibition studies provide no evidence for the antigenic epitope bound by the 121F antibody being carbohydrate in nature. Although the native antigen is blocked at its N-terminus, amino acid analysis of a hydroxylamine generated peptide disclosed a striking relationship between the osteoclast antigen recognized by the 121F monoclonal antibody and manganese and iron superoxide dismutase. Therefore, in addition to serving as a distinguishing cell type–specific marker for osteoclasts, this cell surface glycoprotein may function directly in osteoclast-mediated bone resorption.

Key words: osteoclast, membrane glycoprotein, superoxide dismutase

Osteoclasts are the major cell type responsible for bone matrix degradation. These multinucleated cells are thought to derive from blood-borne precursors related to cells of the mononuclear phagocyte family [1]. However, the exact developmental relationship between circulating mononuclear cells, tissue macrophages, and osteoclast precursors in hematopoietic tissues is not clear. Moreover, there is little yet known about the osteoclast plasma membrane or about modifications of its constituents which may be controlled by physiological or developmental mechanisms. Cell surface molecules which are either uniquely present or preferentially expressed at high levels by osteoclasts are candidates for structural markers, or functional and/or regulatory mediators of osteoclast activities, such as bone resorption. Osteoclasts possess a specialized plasma membrane ruffled border juxtaposed to the bone surface [2,3]. Enzymes which are postulated to contribute to the acidification and calcium translocation associated with the resorption process have been identified in the ruffled border or plasma membrane of osteoclasts. Baron et al. [4] have reported that an antibody cross-reactive with the H^+, K^+-ATPase 100 kDa lysosomal protein binds to the avian osteoclast ruffled border region. Other investigators have identified Mg^{++}-ATPase [5], vacuolar

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H^+-ATPase [6,7,8], and Ca^{++}-ATPase [9] in association with the avian osteoclast plasma membrane. Additional osteoclast surface markers include a presumed calcitonin receptor based on specific, high-affinity calcitonin binding to rat osteoclasts [10], as well as an osteoclast plasma membrane-associated vitronectin receptor [11] that may play a role in cell-matrix interactions. Aside from these advances, there remains much about the components of the osteoclast plasma membrane and their modifications during osteoclast cytodifferentiation which has not been characterized.

As one approach to the identification of osteoclast plasma membrane components, a library of monoclonal antibodies directed against osteoclasts has been developed [12]. A number of these antibodies appear to be osteoclast-specific or expressed in significantly greater abundance on osteoclasts when compared with related in vitro generated marrow-derived giant cells. In previous studies one monoclonal antibody, designated 121F, was reported to recognize an antigen restricted to the osteoclast plasma membrane. When the cell specificity of this antigen was examined by immunohistochemical staining on isolated osteoclasts, on closely related marrow-derived giant cells, and on cells within a variety of tissues, the antigen was only observed on osteoclasts [12]. The 121F antibody was reported to recognize 96 kD, 91 kD, and 45 kD species on western blots which probed detergent extracts of isolated osteoclasts [12], or solely the 96 kD species when the antigen was previously partially purified by immunoaffinity protocols [13]. The relationship between these species was not fully understood at that time; however, more recent studies have shown that these and other degradative fragments represent proteolytic products of the labile osteoclast membrane component reported here. Due to the inherently high proteolytic activity associated with osteoclasts [14], we have modified the past antigen isolation scheme to currently include a complex protease inhibitor mixture in order to avoid artifactual antigen degradation [15].

Expression of the antigen recognized by the 121F monoclonal antibody has also been studied in an in vitro marrow precursor culture system, as well as in an in ovo implant system. This antigen can be induced and maintained on giant cells which form in vitro in the presence of osteoblast conditioned media. In addition, the antigen is expressed on giant cells formed in ovo in response to resorbable substrates but not found on giant cells formed in response to nonresorbable materials [16,17]. The latter studies suggested correlations between antigen induction and both ruffled border formation and the ability of such cells to resorb bone particles. Thus, the antigen recognized by the 121F monoclonal antibody appears to be a developmentally regulated cell component with some role in osteoclast function, but whose involvement in bone resorption has not yet been fully defined.

This report describes the purification and characterization of the plasma membrane glycoprotein identified by the osteoclast-directed 121F monoclonal antibody. This component has been visualized on SDS-polyacrylamide gels as a high molecular weight protein of greater than 200 kDa in its unreduced form and 150 kDa under disulfide bond reducing conditions. The carbohydrate content, nature of the epitope recognized by the monoclonal antibody, and distribution of this protein in marrow-derived multinucleated giant cells are described. Although the intact isolated glycoprotein is N-terminally blocked, partial amino acid sequence data from a peptide fragment has revealed an initially unexpected close homology with superoxide dismutase of the manganese and iron family. Since osteoclasts demonstrate significant levels of superoxide dismutase activity which increase when resorption activity is elevated [18], this enzyme could function to rapidly convert toxic superoxides, generated at the bone matrix-cell interface during active osteoclast resorption [19], into less toxic H_2O_2, which would be further decomposed into H_2O and O_2 by catalase [20]. Therefore, the sequence homology between this plasma membrane glycoprotein and superoxide dismutase provides the first insight into a proposed mechanism for the physiological function of this osteoclast membrane component in bone resorption.

MATERIALS AND METHODS

Cell Isolation and Culture

Osteoclasts were isolated by a modification of the method cited by Oursler et al. [12]. Briefly, 15 White Leghorn chick hatchlings, maintained on a low calcium ($< 0.1\%$ calcium) diet (Purina) for a minimum of 28 days, were euthanized for each osteoclast preparation. The tibias and humeri were dissected free of soft tissue. Marrow was removed by flushing the marrow cavities repeatedly with calcium and magnesium

(Ca,Mg)-free Tyrode's balanced salt solution, pH 7.4 (TBSS), with a 3 cc syringe fitted with an 18 guage needle. The marrow was used to prepare marrow-derived giant cells [16] (see below). Subsequent steps were at 4°C or on ice. Marrow-depleted bones were split longitudinally in Hanks' balanced salt solution (HBSS) (GIBCO), pH 7.4, then agitated by vigorous shaking and twice rinsed with Ca,Mg-free TBSS. Released cells from the pooled rinses (part 1) were reserved in Ca, Mg-free TBSS on ice while bones were treated with 0.5 mg/ml collagenase (Worthington Biochemical Corp.) in TBSS at 37°C for 30 minutes. Cells were released from the bones by repeated agitation and rinsing as above and were added to the cells from part 1 of the cell preparation. These cells were sequentially filtered through 350 μ, 110 μ, and 85 μ nylon mesh filters (Nitex) and then pelleted at 200*g* for 10 minutes. Cell pellets were resuspended in 60 ml of 35% Percoll (Pharmacia) in HBSS, overlaid in 6 tubes with ⅕ this volume of HBSS, and centrifuged at 440*g* for 20 minutes at 4°C. The top layer and interface, containing osteoclasts, were aspirated off, pooled, diluted with HBSS, and then pelleted at 300*g* for 10 minutes. These cell preparations were either used directly for enzyme-linked immunosorbent assay (ELISA) or for fluorescent activated cell sorting, or else stored as a cell pellet at −70°C.

Marrow mononuclear cells obtained from the flushed bone marrow cavities of 15 chicks (as described above) were filtered sequentially through 350 μ and 110 μ nylon filters (Nitex), centrifuged at 1,180*g* for 7 minutes, and the pellets resuspended in 60 ml of 1.67% heparin and 50% fetal calf serum in Ca,Mg-free TBSS. 15 ml of the cell mixture was carefully overlaid onto each of 4 tubes containing 0.5 ml heparin and 15 ml Ficoll-Paque (Pharmacia), centrifuged at 420*g* for 30 minutes, and 14 ml removed from each tube at the buffy white cell layer region to another set of tubes, each containing 0.5 ml heparin and 35 ml Ca,Mg-free TBSS. Cells were spun at 1,180*g* for 7 minutes, washed twice by resuspension in Ca,Mg-free TBSS and centrifugation at 1,180*g* for 5 minutes, and resuspended in alpha minimal essential media (alpha-MEM) containing 10% fetal calf serum (FCS) (GIBCO) and 1% antibiotic-antimycotic (GIBCO). 50×10^6 marrow cells were plated per 100 mm tissue culture dish (Falcon) and cultures were maintained at 37°C in a 95% air/5% CO_2 moist environment. Media were replaced every other day, and by the second to fourth day of culture, cells had begun to spontaneously fuse to form marrow-derived multinucleated giant cells. Beyond day 7, giant cells constituted at least 80–90% of the adherent cells. Cells from day 12 cultures were gently rinsed several times with HBSS, scraped off dishes using a rubber policeman, and stored as a pellet under the same conditions described for storage of osteoclast pellets.

Fluorescent Activated Cell Sorting

Following 35% Percoll fractionation, osteoclasts were fixed in suspension with 1% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, rinsed with PBS, then blocked for nonspecific antibody binding with 1% bovine serum albumin (BSA) and 10% fetal calf serum (FCS) in PBS (block) for 60 minutes. Cells were then reacted for 60 minutes with 121F monoclonal antibody mouse ascitic fluid at a 1:200 dilution in block, rinsed with PBS, and incubated with FITC conjugated goat antibody directed against mouse immunoglobulins (Cappell Laboratories) for an additional 60 minutes. Following washing with PBS, cells were analyzed on a Becton-Dickenson fluorescent activated cell sorter (FACS) using a 200 μ aperture.

Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunoassays were conducted similarly to that reported in Oursler et al. [12], with the following modifications. All steps were performed at ambient temperature. Plates were rinsed several times over 30 minutes with ELISA wash, consisting of 0.02% Tween 20, 0.15 M NaCl in 0.02 M imidazole buffer, pH 7.4, then incubated for 1 hour in block solution containing 1% BSA (Sigma Chemical Co.) and 10% FCS (GIBCO) in PBS. Block was replaced with 121F monoclonal antibody (mouse ascitic fluid) diluted 1:4,000 in block solution (75 μl/well) and the plates were incubated for 1 hour. Wells were rinsed 3 times with ELISA wash solution, then incubated for 1 hour with 75 μl/well of 1 μg/ml peroxidase conjugated affinity purified goat anti-mouse IgG + IgM (H&L) in block solution (Kirkegaard and Perry Laboratories, Inc.). After washing 8 times, wells were incubated 1 hour in the dark with 75 μl of peroxidase substrate mixture containing ABTS (2,2-azinondi-C3-ethyl-benzthiazoline sulfonate) and hydrogen peroxide in cacodylate buffer (KPL). Reaction product was measured by absorbance at 405 nm

in a Dynatech ELISA reader. ELISA specific activity values were determined by normalizing ELISA absorbance readings for protein (mg). For protein determinations, triplicate wells incubated throughout the ELISA steps with PBS only were briefly sonicated (to dislodge protein) in 200 μl 0.005% Triton X-100, 0.075 M NaCl, 1.5 mM $NaHCO_3$, boiled 5 minutes with 100 μl 1N NaOH, and analyzed by the Lowry method [21].

Periodate Treatment and Hapten Inhibition Studies

Acidic periodate cleavage of vicinal hydroxyls was performed according to Woodward et al. [22]. Briefly, isolated osteoclasts were treated for 1 hour at 25°C in the dark with 10 mM sodium meta-periodate (Sigma Chemical Co.) in 0.1 M sodium acetate, pH 4.5. The cells were rinsed with 0.1 M sodium acetate, pH 4.5, blocked for 30 minutes with 1% glycine, rinsed, and dispensed into wells of a 96 well ELISA dish precoated for more than 16 hours with poly-L-lysine. Typically, portions of the 35% Percoll fractionated osteoclast preparation from 15 chicks (ranging from ¼ to the total) were gently resuspended in 2.5 ml HBSS (GIBCO), 50 μl aliquots placed in each well (using wide-bore tips), and the cells spun onto the dish by centrifugation at 50*g* for 5 minutes. Cells were immediately overlaid with 150 μl of PBS containing 1% paraformaldehyde and 0.00025% glutaraldehyde for 15 minutes at room temperature, the fixed cells were rinsed 3 times with HBSS, and the cells were either assayed directly by ELISA, or alternatively, stored overnight at 4°C in HBSS containing 1.03 mM PMSF prior to ELISA. Monosaccharide hapten inhibition studies were performed by premixing monosaccharides (Sigma Chemical Co.) with 121F monoclonal antibody ascitic fluid in ELISA blocking solution for 30 minutes at 37°C (final sugar concentration 0.5 M; final ascitic fluid dilution 1:20,000). Cells spun and fixed on ELISA dishes as above were assayed by ELISA using the monosaccharide pretreated 121F antibody and compared with results obtained using a similar 121F antibody dilution lacking sugar.

Antigen Purification

Sepharose CL6B (Pharmacia) beads were activated at high pH with cyanogen bromide [23], washed well with 0.05 M citrate buffer, and incubated with 121F ascitic fluid (1 ml antibody per 5 ml settled matrix volume) overnight at 4°C with mixing for coupling. The matrix was blocked for 4 hours by mixing with an equal volume of 2 M glycine, then washed repeatedly with PBS. Coupled matrices were stored at 4°C in PBS containing 0.01% sodium azide.

Osteoclast extracts were prepared by disrupting 35% Percoll separated osteoclasts in ice-cold CHAPS extraction solution consisting of 0.15 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, 0.5% CHAPS (3-[(Cholamidopropyl)-dimethylammonio]-1-propanesulfonate), pH 7.4, containing the following protease inhibitors (Sigma Chemical Co.): 0.03% soybean trypsin inhibitor, 0.1 M epsilon-amino-N-caproic acid, 0.002 M PMSF, 0.4 trypsin inhibitor units/ml aprotinin, and 10 μg/ml each of leupeptin, chymostatin, antipain, and pepstatin. The cell extract was microcentrifuged at 10,000 rpm for 5 minutes at 4°C. After overnight incubation at 4°C with the immunomatrix, the matrix was repeatedly washed with chilled PBS containing 0.002 M PMSF and 0.001 M EDTA (wash solution) and the matrix collected by centrifugation at 50*g* for 10 minutes. The matrix was poured into a small column and eluted at room temperature with a continuously increasing concentration (0–8 M gradient) of deionized ultrapure urea (International Biotechnologies, Inc.) in PBS. All fractions were collected and dialyzed separately overnight at 4°C against freshly prepared wash solution. Aliquots of each fraction were tested by ELISA for the presence of 121F antibody-reactive antigen by mixing with $\frac{1}{100}$ volume of 25% aqueous glutaraldehyde (Sigma Chemical Co.), dispensing equivalent portions (100 μl) per well of a 96 well poly-L-lysine precoated and rinsed ELISA dish, and drying samples onto the dish overnight at 40°C. Fractions reactive with the 121F antibody were pooled and concentrated on ice using an Amicon cell concentrator.

Immunoaffinity purified antigen was heated to 95°C for 10 minutes in the presence or absence of 5% β-mercaptoethanol, cooled, applied to a Sepharose CL6B column (Pharmacia/LKB) and chromatographed in 30% acetonitrile. Dialyzed fractions were analyzed by ELISA as previously described for the immunoaffinity column fractions.

Bone and Cell Extractions for ELISA

One-quarter of a bone, either immediately after its removal from the chick or following marrow removal, was extracted in 2 ml of ice-

cold CHAPS extraction solution (as above), homogenized with a Brinkman Polytron on ice for 60 minutes, and particulates removed by microfuge centrifugation at 10,000 rpm for 5 minutes at 4°C. Supernatants were rapidly desalted by centrifuging through Bio-Gel P6DG (BioRad Laboratories) which had been equilibrated with PBS, mixed with 1/100 volume of 25% aqueous glutaraldehyde (Sigma Chemical Co.), and 100 μl portions plated per well of a poly-L-lysine precoated 96 well ELISA dish. Plates were dried overnight at 40°C prior to ELISA.

Osteoclasts were solubilized in ice-cold CHAPS extraction solution, microcentrifuged, and the supernatants desalted as described above. Portions were fixed, plated, dried, and analyzed by ELISA as for bone extracts and purified antigen.

SDS-Polyacrylamide Gel Electrophoresis and Isoelectric Focusing

Electrophoresis was carried out by the method of Laemmli [24] using a 5% to 20% polyacrylamide linear gradient resolving gel with a 5% stacking region. Protein was visualized using the silver staining method of Morrissey [25]. Molecular weight markers (BioRad) were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). Purified antigen was analyzed by isoelectric focusing according to the method of O'Farrell [26], with silver stain visualization [25].

Western Blot Analysis

Frozen marrow-derived giant cells and osteoclasts were extracted with CHAPS extraction solution as above (typically 1/8 of an osteoclast preparation from 15 chicks or 1/4 of the harvested marrow-derived giant cells from a 100 mm tissue culture dish per ml extraction solution). Portions were mixed equally with electrophoresis sample buffer containing the following protease inhibitors: 1.3 mM EDTA, 80 mM epsilon-amino-N-caproic acid, 1.1 mM PMSF, 0.4 trypsin inhibitor units/ml aprotinin, and 10 μg/ml each of leupeptin, chymostatin, antipain and pepstatin. Samples were electrophoresed by SDS-PAGE and the proteins were transferred to nitrocellulose by the method (omitting methanol) of Towbin et al. [27], using a Genie electrotransfer unit (Idea Scientific). The blots were blocked for 16 hours at 4°C with 5% nonfat dry milk in PBS, rinsed with PBS, reacted for 24 hours at 37°C with 121F monoclonal antibody diluted 1:100 in PBS with 0.05% Tween 20 (PBST), rinsed 3 times over 1 hour at 4°C with PBS, and further reacted for 2 hours at 37°C with alkaline phosphatase conjugated goat anti-mouse IgG antibody (Sigma Chemical Co.) diluted 1:1,000 in PBST. Following rinsing 3 times over 1 hour at 4°C with PBST, the blots were further reacted for 2 hours at 37°C with a 1:1,000 dilution in PBST of alkaline phosphatase conjugated rabbit anti-goat IgG antibody (Sigma Chemical Co.). After a 60 minute wash with 3 changes of 0.1 M Tris buffered saline, pH 7.9, blots were incubated for 30 minutes with napthol anilid-Saur-Mix (AS-MX) phosphate and Fast Blue RR salt (Sigma Chemical Company histochemical kit), to visualize alkaline phosphatase activity.

Carbohydrate Analysis

Concanavalin A (Con A), Ricin Agglutinin (RCA), and Wheat Germ Agglutinin (WGA) lectin affinity matrices were purchased from Sigma Chemical Company. Antigen purified by immunoaffinity chromatography was diluted in loading buffer (0.01 M Tris, 0.001 M $MgCl_2$, pH 7.9), applied to the columns, the columns washed well with loading buffer, and any bound species eluted from the Con A, RCA, and WGA columns with 0.5 M alpha-methyl-mannoside, lactose, or N-acetyl glucosamine, respectively. Column elution profiles were monitored by absorbance at 280 nm. For endoglycosidase treatment, immunoaffinity purified antigen was denatured in 0.2% SDS and 2% β-mercaptoethanol with boiling [28]. Nonidet P-40 detergent (Sigma Chemical Co.) was added to a final concentration of 2% to exchange for SDS. Antigen was treated with either Endoglycosidase H or Glycopeptidase F (Boehringer Mannheim Biochemicals) at 37°C for 16 hours. Trifluoromethanesulfonic acid treatment of purified antigen was carried out by the method of Edge et al. [29]. SDS-PAGE analysis of the products was visualized by Coomassie blue staining [30]. Periodate oxidation was performed by the method of Bobbitt [31]. In all cases, fetuin, rabbit IgG, and ovalbumin were analyzed in parallel as positive controls for carbohydrate alterations (data not shown).

Amino Acid Sequencing

Initial attempts to obtain amino acid sequence data from the antigen which can be recognized and purified with the 121F antibody

indicated that the antigen was N-terminally blocked. Therefore, hydroxylamine peptides were generated from purified antigen as follows. Immunoaffinity purified antigen was subjected to reducing SDS-PAGE as above, the 160 to 145 kDa region was excised from the gel, and the antigen was electroeluted in an electroelution (ISCO) apparatus for 16 hours by the method of Kelly et al. [32]. This material was dialyzed against 0.1 M Tris, pH 7.4, for 2 days with 4 changes of buffer before its cleavage by hydroxyalamine for 48 hours at room temperature as outlined by Mahboub et al. [33]. The digest was electrophoresed by SDS-PAGE and transferred to an Immobilon PVDF (Millipore Corp.) membrane as recommended by the manufacturer. Following a 2 hour transfer, the membrane was stained with 0.1% Ponceau S in 1% acetic acid, and destained with 1% acetic acid as described by Aebersold et al. [34]. A number of bands, including a 30 kDa region, were excised for amino acid sequencing attempts by automated Edman degradation using an Applied Biosystems Model 470A amino acid sequencer.

RESULTS

Antigen Specificity and Identification

As a prelude to isolating and biochemically characterizing the osteoclast antigen recognized by the 121F monoclonal antibody, it was important to confirm its specific association with osteoclasts in the bone-derived cell preparations. Previous ELISA and immunohistochemical studies had shown that the 121F monoclonal antibody (MAb) was highly reactive with osteoclasts in cell preparations or frozen sections [12]. These findings were extended to a larger population analysis by fluorescent activated cell sorting (Figure 1). Preparations of partially purified osteoclasts were analyzed for surface antigen recognized by the 121F monoclonal antibody (MAb). As shown in panels a and b, light scatter and fluorescent profiles of the total cell population demonstrated that a majority of the cells were small to mid-range in size and displayed a broad non-descript fluorescent pattern. Panels c and d illustrate the light scatter and fluorescent pattern, respectively, of the mononuclear cell population. Following 35% Percoll fractionation, the osteoclast population contained significant numbers of small mononuclear cells. The 121F antibody reactivity with these cells was minimal (panel d). Panels e and f represent the light scatter and 121F antibody reactivity, respectively, of the mid-range cells composed of larger mononuclear cells and smaller multinucleated cells with few nuclei. These cells exhibited a low, but significant, level of 121F antibody binding. The population of large multinucleated cells (panels g and h), however, demonstrated a strong reaction with the 121F MAb (panel h). These data therefore confirmed that the 121F MAb predominantly recognized the larger cells in osteoclast preparations, with little binding to contaminating mononuclear cells.

The molecular specificity determined by Western blot was reevaluated from that described in earlier reports [12], by employing osteoclast extracts and marrow-derived giant cell extracts prepared with more extensive protease inhibitor supplementation to avoid artifactual antigen degradation. Electrophoresis of such extracts, followed by transfer to nitrocellulose and probing with the 121F MAb, confirmed the presence of a single 121F MAb–reactive band in osteoclasts (Figure 2B, lane 2). This antigen migrated as a 150 kDa species under reducing conditions. The absence of any reactive species in the marrow-derived giant cell population (Figure 2B, lane 1), when comparable amounts of protein were analyzed, demonstrated that the 121F MAb–reactive antigen was either absent from these closely related cells or significantly lower in abundance.

Antigen Purification

Fractions eluted from the 121F MAb immunoaffinity column were analyzed for the osteoclast antigen by ELISA as shown in Figure 3. Those fractions (21–24) demonstrating strong reactivity with the 121F MAb were pooled, concentrated by Amicon filtration, and chromatographed on a Sepharose CL6B column in 30% acetonitrile. The Sepharose CL6B column profile shown in Figure 4 pertains to a sample which was pretreated by heating at 95°C in β-mercaptoethanol. This method of sample preparation gave consistent and reproducible column profiles. Both the protein profile, as monitored by absorbance at 280 nm, and the 121F MAb reactivity, as measured by ELISA, indicated that the Sepharose CL6B chromatographic step was effective in increasing the purity of the antigen recognized by the 121F antibody (Table I). Column fractions that reacted with the 121F antibody (fractions 13–15) were pooled and analyzed by SDS-PAGE, revealing a major band at 150 kDa when the sample was analyzed on disulfide reducing gels (Figure 5A, lane 1). In analo-

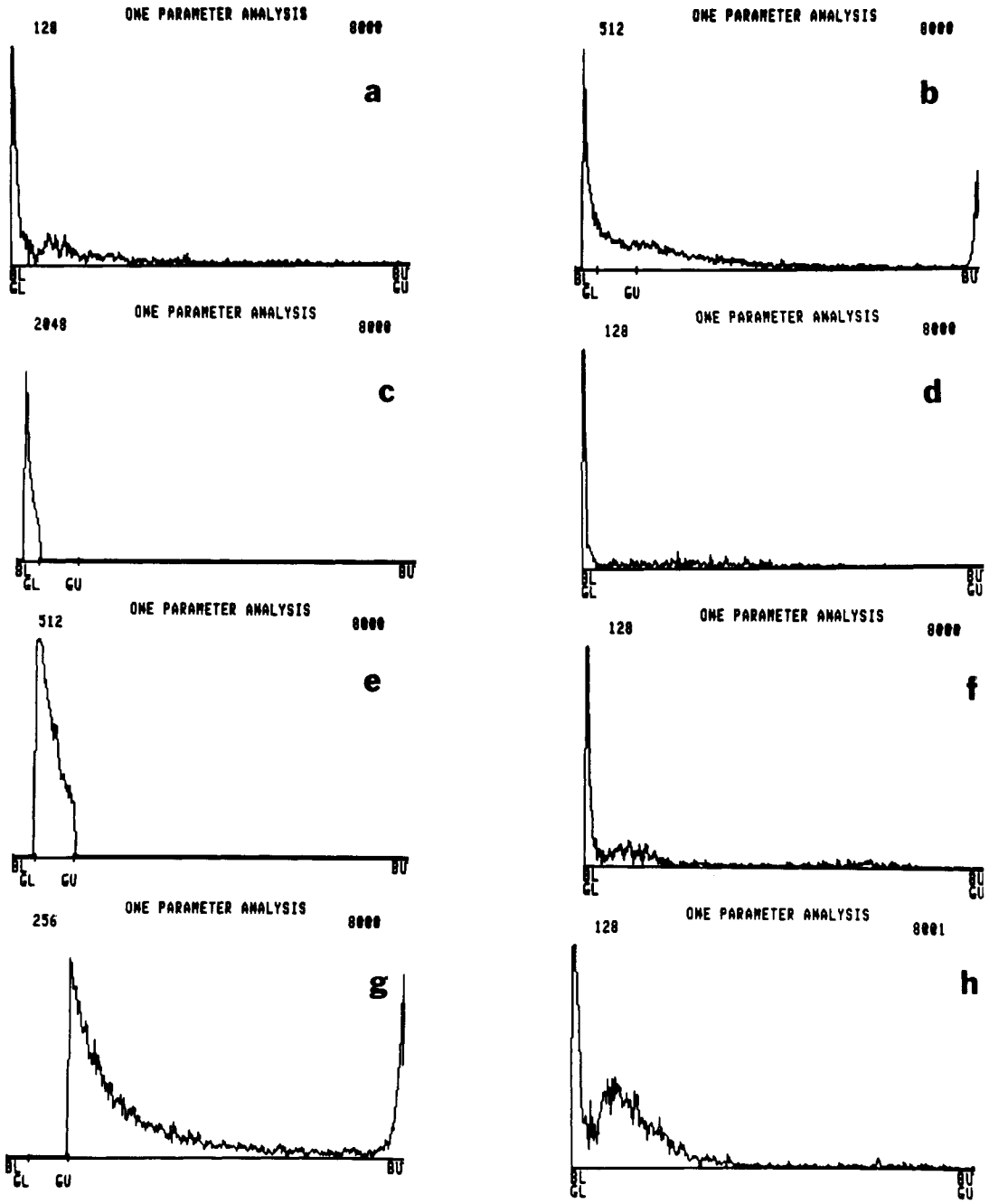


Fig. 1. Fluorescent activated cell sorting of isolated osteoclasts. In **a, c, e,** and **g** the x-axis represents the forward angle light scatter which is correlated to the size of single cells. In **b, d, f,** and **h** the x-axis represents the channel number which corresponds to the amount of fluorescent label. In all panels, the y-axis represents the cell number in each channel. Panels a and b demonstrate the size distribution and 121F MAb reactivity, respectively, of the entire 35% Percoll separated osteoclast cell preparation. Panels c and d show the size and 121F MAb reactivity, respectively, of the smallest 5% of the cells present. Panels e and f display the size and 121F MAb reactivity, respectively, of the mid-size range cells (representing the 5% to 15% size distribution class). Panels g and h depict the size and 121F MAb reactivity of the largest cells present (the 15% to 100% size class; up to 200 μ in diameter). These profiles represent fluorescence due to both endogenous and secondary antibody activity.

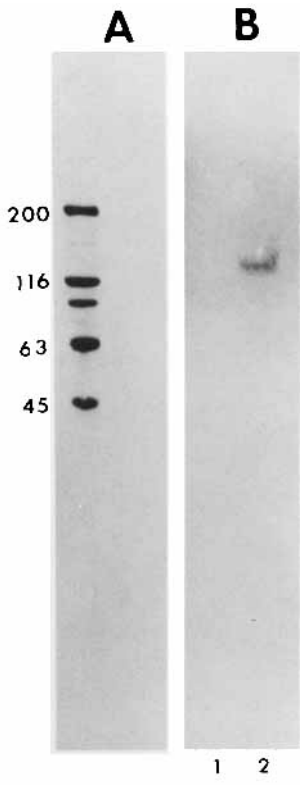


Fig. 2. SDS-PAGE and Western blot analysis. **A:** Molecular weight markers transferred to nylon-backed nitrocellulose membrane (Micron Separations, Inc.) and Ponceau S stained as given in the Methods. **B:** Western blot of extracts of marrow-derived giant cells (lane 1) and osteoclasts (lane 2) probed with the 121F MAb, followed by alkaline phosphatase conjugated goat anti-mouse IgG.

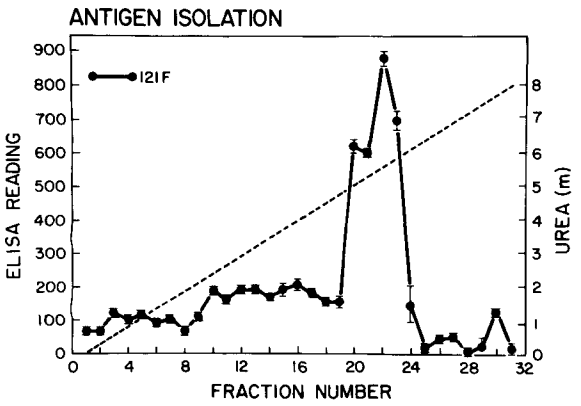


Fig. 3. Immunoaffinity column chromatography. CHAPS extracts of osteoclasts were bound to a 121F MAb immunoaffinity column, eluted with a 0–8 M linear urea gradient, and the fractions individually dialyzed before ELISA as described in Methods. A peak of 121F MAb–reactive material was reproducibly obtained from the immunoaffinity matrix in the elution region of 5–6 M urea.

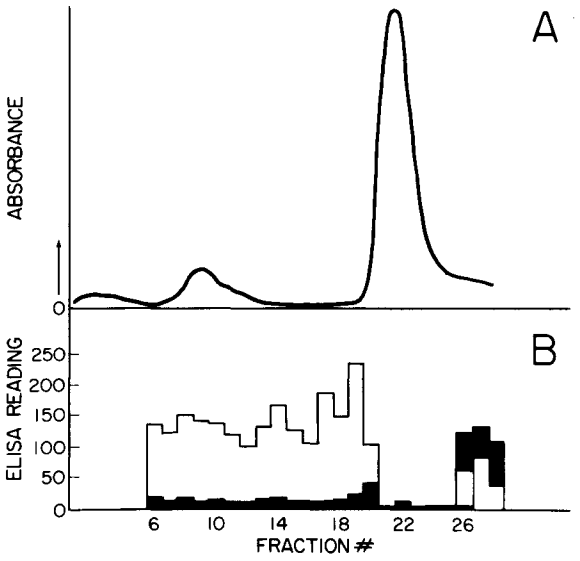


Fig. 4. Sepharose CL6B column chromatography. Immunoafinity isolated antigen (Figure 3, fractions 20–23) were pooled, concentrated by Amicon filtration, pretreated with β-mercaptoethanol at 95°C, and chromatographed on a Sepharose CL6B column. Antigen was eluted under isocratic conditions in 30% acetonitrile and the eluted protein was monitored by absorbance at 280 nm (**panel A**). Equivalent portions of each fraction were individually dialyzed and analyzed for 121F MAb reactivity by ELISA as described in Methods (**panel B**). The open bars represent 121F MAb reactivity, whereas the closed bars describe binding of the goat anti-mouse IgG alone. (Note the apparent residual 121F MAb from the immunoaffinity step which is detectable at the far right).

gous samples prepared in the absence of β-mercaptoethanol, the antigen was present as a single larger species of greater than 200 kDa (Figure 5A, lane 2). Isoelectric focusing of the antigen after Sepharose CL6B chromatography also revealed a single purified species, having a pI of 6.95 (Figure 5B).

Quantitation of Purification

Table I lists the stages in the antigen purification. There was a continual enrichment for 121F MAb–reactive protein throughout the purification stages, as demonstrated by an increase in ELISA specific activity of more than 2×10^6 fold when intact bone was compared with Sepharose CL6B purified antigen. This high degree of purification is not surprising when the complexity of the starting bone tissue is considered. There may be a significant drop in total 121F MAb–reactive protein measured by ELISA after urea elution from the immunoaffinity matrix as a result, at least in part, of a loss of antigenicity following exposure to urea. This apparent loss was reversed following size exclusion chro-

TABLE I. Antigen Purification*

	Relative total ELISA absorbance	Yield (%)	ELISA specific activity	Enrichment factor
Intact bone	16,500	100	0.0004	1
Bone with marrow removed	11,070	67	0.0012	3
Isolated cells before Percoll	2,354	14	1.619	4,048
35% Percoll top, interface	1,408	8	3.0	7,500
121F MAb immunoaffinity chromatography	17	0.1	42.4	106,000
Sepharose CL6B chromatography	63	0.4	1050.0	2,625,000

*At each step in the purification of the antigen from 15 chicks, CHAPS extracts of bone or isolated cells were prepared and assayed for 121F MAb reactivity by ELISA as described in Methods. Portions of purified antigen were similarly analyzed by ELISA. Absorbance readings were extrapolated to derive totals for each step, and also expressed as specific activities based on protein determinations for each sample. Antigen yield was set at 100% for the total ELISA reading associated with intact bone and the other samples expressed as a percentage of this based on their ELISA determinations. Specific activities were compared to derive antigen enrichment factors for each step in comparison with intact bone.

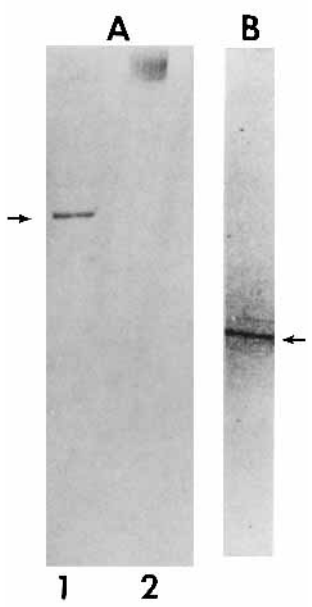


Fig. 5. SDS-PAGE and isoelectric focusing of purified antigen. A: Silver stained profile of the 150 kDa (arrow) Sepharose CL6B purified 121F MAb–reactive osteoclast antigen analyzed in the presence (lane 1) and absence (lane 2) of β-mercaptoethanol. The unreduced molecule migrates as a protein larger than 200 kDa. Isoelectric focusing of the purified 121F MAb–reactive osteoclast antigen with silver stain visualization, demonstrating the presence of a single species with a pI point of 6.95 (arrow).

matography, which suggested some antigen renaturation. Thus, the actual yield of antigen reactive with the 121F antibody may be significantly greater than reported on the basis of ELISA analysis alone. The purification scheme outlined here has been used repeatedly with consistently reproducible results. More recently, similar results have been obtained using Sephadex G-200 (Pharmacia) purified 121F MAb in the immunoaffinity procedure (data not shown).

Epitope Analysis

Since most membrane proteins are glycoproteins, we have investigated the potential role of carbohydrate moieties in the osteoclast antigen–121F MAb interaction. Mild periodate oxidation at acid pH, known to cleave terminal carbohydrate vicinal hydroxyl groups without altering polypeptide chains [22], did not destroy the antibody recognition site found on intact isolated osteoclasts as measured by ELISA specific activities (20.78 ± 0.80 for periodate treated versus 17.27 ± 0.90 for control). This suggested that the epitope is either a peptide sequence or a carbohydrate not antigenically altered by the periodate treatment. Furthermore, hapten inhibition studies (Table II) demonstrated no successful inhibition of antibody-antigen recognition by a spectrum of monosaccharide sugars. These results are consistent with the Western blot identification of only one protein reactive with the 121F MAb.

Carbohydrate Analysis of Purified Antigen

Lectin affinity chromatography of the immunoaffinity purified antigen (Figure 6) resulted in the 150 kDa protein being bound to WGA. Such binding indicated that this glycoprotein contains complex N-linked moieties. The failure of the protein to bind Con A or RCA suggested that no unprocessed high mannose-type or β-D-galactose carbohydrates were present. When column fractions were concentrated and subjected to SDS-PAGE with silver stain analysis, a 150 kDa protein band was observed in samples which corresponded with the recorded protein peaks (data not shown). To investigate the degree and

TABLE II. Hapten Inhibition of Antibody Recognition of Osteoclast Cell Surface*

Monosaccharide	Percent of 121F antibody binding on treated osteoclasts compared to controls
Arabinose	108 ± 3
Fucose	118 ± 5
Lactose	118 ± 6
Sucrose	119 ± 2
Glucose	128 ± 2
Glucosamine	132 ± 7
N-acetyl-glucosamine	111 ± 3
3-0-methyl-D-glucose	105 ± 5
Mannose	135 ± 5
Mannosamine	131 ± 3
N-acetylmannosamine	139 ± 1
α-methylmannoside	122 ± 6
Galactose	116 ± 5
Galactosamine	125 ± 5
N-acetyl-galactosamine	113 ± 6
N-acetyl-neuraminic acid	104 ± 5
None	100

*Monosaccharides were individually preincubated with 121F MAb before being used in ELISA analysis of osteoclasts as described in Methods. Control trials employed similarly diluted 121F MAb without sugar. Results are expressed as the percent ratio of experimental to control ELISA readings, with no sugar pretreatment set at 100%. Errors are based on standard deviations.

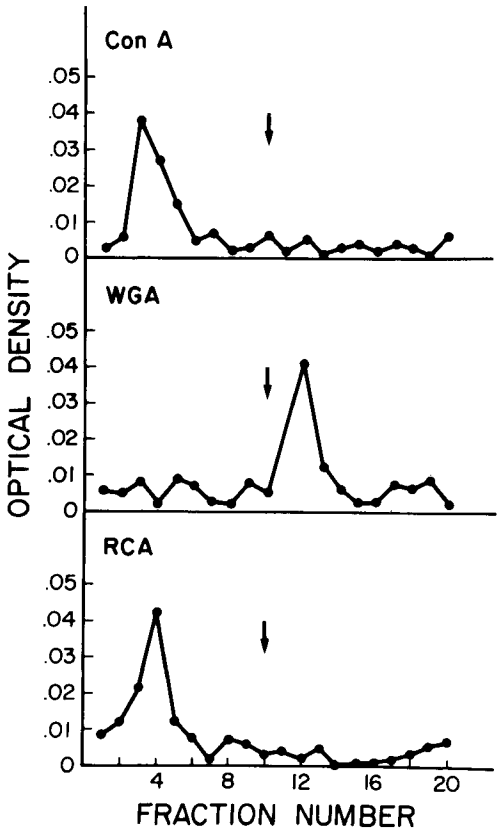


Fig. 6. Lectin affinity chromatography of immunoaffinity purified antigen. Solid lines trace the absorbance at 280 nm (y-axis) of fractions eluted from the lectin columns. Arrows indicate initiation of 200 mM sugar elution as follows: Con A: alpha-methylmannoside; WGA: N-acetylglucosamine; RCA: lactose.

nature of glycosylation of the antigen, treatment with Endoglycosidase H or Glycopeptidase F was performed, followed by SDS-PAGE and Coomassie blue staining. Analysis of these digests (Figure 7) demonstrated a shift in the molecular weight of this protein from 150 kDa to 123 kDa after Glycopeptidase F treatment (lane D). Again, this indicated the presence of complex N-linked sugars. In addition, a higher molecular weight species was lost upon treatment. This protein may represent antigen possessing either different glycosylation levels or partially processed carbohydrates. The lack of a noticeable shift after treatment with Endoglycosidase H (lane C) suggested that none of the carbohydrate species were high mannose-type N-linked sugars. Since treatment with trifluoromethanesulfonic acid (TFMSA, lane E), caused the protein to migrate similarly to that of the Glycopeptidase F treated samples, there must be no O-linked sugar species (Figure 7, lane E). The faint lower molecular weight bands apparent in lanes B and C are most likely contaminating proteins of unknown origin. Amino acid sequence analysis of amino-terminal ends of these contaminants have not revealed their identities (data not shown).

Hydroxylamine Fragment Amino Acid Sequence

Initial attempts to obtain amino acid sequence data from the antigen recognized by the 121F MAb indicated that the N-terminus was blocked. After partial purification of the antigen on a 121F MAb immunoaffinity column, the antigen was further purified on SDS-PAGE and the 140–160 kDa region electroeluted. Electroeluted antigen was exposed to hydroxylamine to generate numerous peptide fragments which were then separated by SDS-PAGE (data not shown). Amino acid sequence analysis of one 30 kDa hydroxylamine fragment (Figure 8A) demonstrated a strong homology with the N-terminus of the mature form of manganese (Mn) and iron (Fe) superoxide dismutase (SOD) family

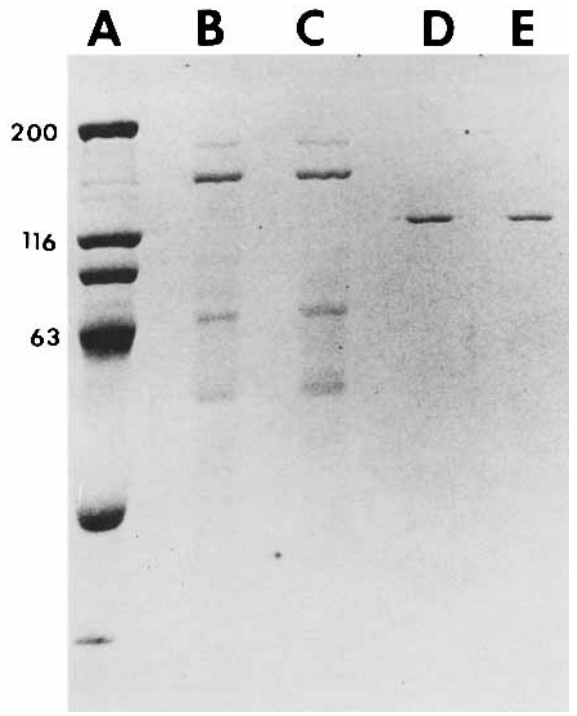


Fig. 7. Glycosidase sensitivity of the immunoaffinity purified antigen. **Lane A:** Molecular weight markers. **Lane B:** Untreated control. **Lane C:** Endoglycosidase H treated sample. **Lane D:** Glycopeptidase F treated sample. **Lane E:** TFMSA treated sample. Samples were analyzed by SDS-PAGE and Coomassie blue staining.

members. A comparison of the first fifteen amino acids of the hydroxylamine fragment of the osteoclast antigen with the Mn and Fe SODs demonstrated a marked degree of homology between these apparently dissimilar cell components. Of these first 15 amino acids in the antigen fragment, there were no residues which were not shared with at least one of the SODs. The included invariant SOD amino acid positions, proline at position 5, leucine at position 7, alanine at position 13, and leucine at position 14, were 100% conserved in the osteoclast antigen. The degree of fragment homology between the purified antigen and these SODs is presented in Figure 8B. Antigen fragment homology with these SODs ranged between 82% and 47%, whereas homologies among known SOD proteins ranged between 89% and 31% [35].

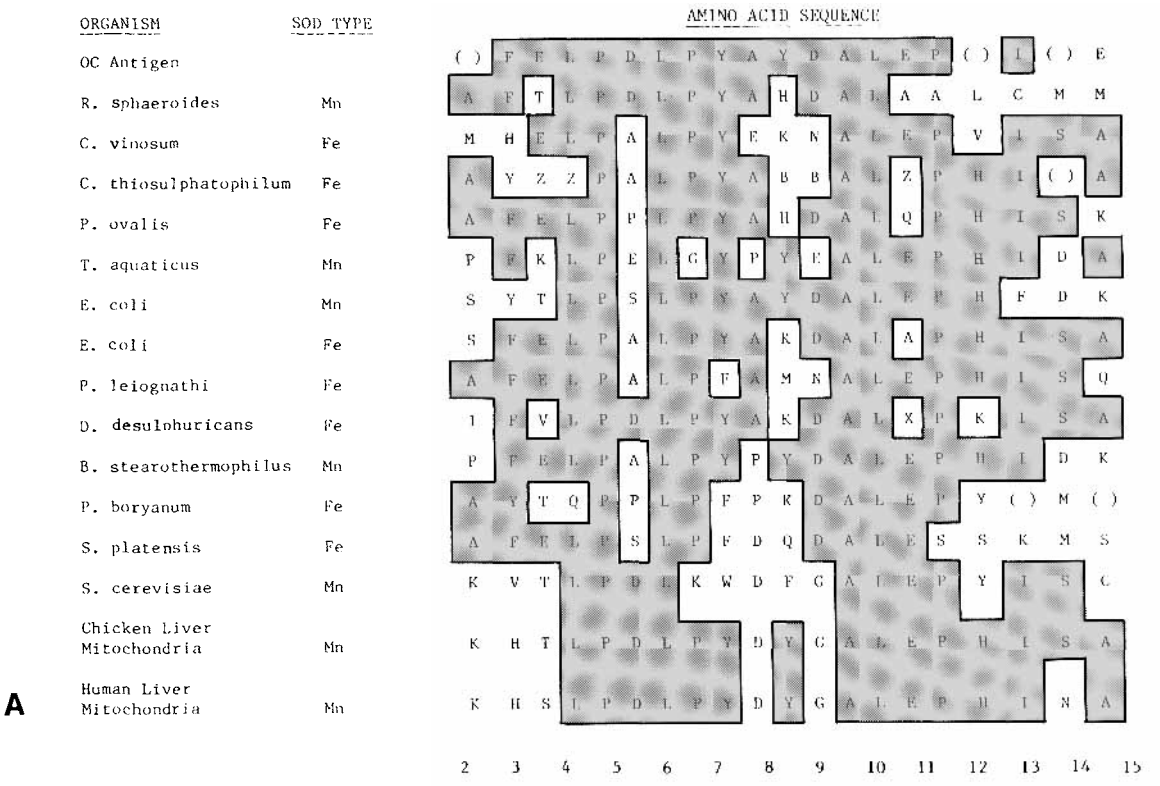
DISCUSSION

A clear understanding of osteoclast physiology, differentiation, and regulatory events has been hampered by the inability to isolate pure populations containing large numbers of authentic osteoclasts. Some investigators have circumvented this problem by employing alternative, closely related, and more readily accessible cell types [36,37,38]. Although much can be gained with respect to elucidating various aspects of osteoclast function and regulation by studying other model systems, we have focused our efforts on, first, identifying epitopes restricted to osteoclasts, and then delineating their contribution to this distinct cell type. One advantage of this approach is that the issue of cell purity can be potentially bypassed. As is the case with many other osteoclast isolation protocols, our population is not totally pure. However, based on immunohistochemistry and ELISA analysis [12,13,17], as well as the FACS analysis reported here, a monoclonal antibody has been developed in this laboratory which can distinguish osteoclasts from contaminating cell types. This antibody is therefore valuable in many respects, including its potential for extracting an osteoclast protein from such cell populations. An earlier report presented Western blot data indicating that the 121F monoclonal antibody recognized three gel bands from osteoclasts of 96, 91, and 45 kDa [12]. The absence of these lower molecular weight species in the purified sample and western blot analyses presented in this report are consistent with the supposition that they arose as degradation products of the 150 kDa protein, a not unlikely event since the only protease inhibitor present in the earlier study was PMSF. These and other degradative fragments of the osteoclast antigen are now known to be obtained only when insufficient protease inhibitors are present during its purification (data not shown).

Steps in the antigen purification protocol were monitored by ELISA 121F MAb reactivity, and demonstrated an effective purification of the antigen of over 2 million–fold. However, there was a sharp drop in the yield of antigen recovered during purification. This may be due to an inevitable loss of the protein throughout this procedure, or, in part, the drop may reflect urea-induced or other loss of antigenicity.

When immunoaffinity purified antigen was analyzed by SDS-PAGE, the predominant protein band was the 150 kDa species. However, on occasion, minor contaminating bands were observed in areas where immunoglobulin heavy chains and light chains migrate on SDS-PAGE. These minor bands are not reactive with the 121F MAb on Western blots (data not shown). It is probable that during immunoaffinity urea

A



B

1.	OC Antigen														
2.	R. sphaeroides	Mn	65												
3.	C. vinosum	Fe	65	42											
4.	C. thiosulphatophilum	Fe	53	44	64										
5.	P. ovalis	Fe	76	62	61	61									
6.	T. aquaticus	Mn	65	40	52	47	59								
7.	E. coli	Mn	70	48	50	51	53	54							
8.	E. coli	Fe	76	52	79	75	72	52	55						
9.	P. leiognathi	Fe	70	46	68	65	74	51	51	72					
10.	D. desulphuricans	Fe	76	43	56	59	57	45	46	67	54				
11.	B. stearothermophilus	Mn	82	48	60	54	64	68	67	62	64	50			
12.	P. boryanum	Fe	47	48	48	62	48	39	48	48	42	45	48		
13.	S. platensis	Fe	59	52	50	51	51	44	44	52	59	46	50	52	
14.	S. cerevisiae	Mn	53	31	45	39	38	35	45	43	41	37	38	38	31
15.	Chicken Liver (Mitochondria)	Mn	70	43	59	56	54	57	57	59	54	52	61	48	43	67
16.	Human Liver (Mitochondria)	Mn	70	34	50	54	45	52	55	50	45	46	55	38	38	61	89

Fig. 8. Hydroxylamine fragment amino acid sequence. **A:** A comparison of the amino acid sequence of a 30 kDa hydroxylamine fragment from the 121F MAb–reactive osteoclast antigen with the amino-terminal sequence of Mn and Fe superoxide dismutases [reviewed in 34]. The shaded region emphasizes the common invariant or predominant amino acid residues for each position of the sequence. **B:** Percentage similarity matrix for the antigen and superoxide dismutases. The antigen values were calculated over the sequence lengths shown in 8A. Unidentified residues were not scored at all. The superoxide dismutase values were taken directly from reference 34. Numbers along the top refer to the source of each SOD under comparison and correspond directly to the numbers preceding each sample listed in the figure.

elution, a small amount of 121F monoclonal antibody dissociates from the matrix along with the antigen. This possibility is supported by the detection of mouse antibody that does not comigrate with the antigen recognized by the 121F MAb when Sepharose CL6B chromatographic fractions are analyzed by ELISA. For this reason, and to generally circumvent the problem of minor contaminating protein species, the 150 kDa reduced antigen band was electroeluted from the SDS-polyacrylamide gel before treatment or processing for all amino acid sequence analyses.

Davies et al. [11] have purified a protein from a kidney cell line using a monoclonal antibody which recognizes both osteoclasts and mononuclear kidney cells. This antigen appears to be a member of the integrin family based on amino-terminus sequence data. The research presented here is the first identification and purification of a cell surface protein found on osteoclasts which is undetectable on mononuclear cells and mononuclear-derived polykaryons. By utilizing the 121F monoclonal antibody in an immunoaffinity column, we have purified a cell surface component from isolated osteoclasts which migrates as greater than 200 kDa and as 150 kDa on unreduced and reduced SDS-PAGE, respectively. The purified antigen is reactive with the 121F MAb in Western blots and the osteoclast epitope was shown to be absent from closely related multinucleated cells. Biochemical characterization of the purified antigen has established that it is a high molecular weight cell surface glycoprotein which possesses complex N-linked sugar moieties but little or no O-linked sugars [39,40]. Although the osteoclast antigen is a membrane glycoprotein, the epitope recognized by the 121F MAb on intact cells is not adversely affected by acidic periodate oxidation nor inhibited by simple sugars and so is either a peptide sequence or a carbohydrate group which is not antigenically altered by these treatments. The former is suggested by the sole identification of one protein band on Western blots which is reactive with the 121F MAb.

The amino acid sequence data which suggested homology between the osteoclast antigen and manganese and iron superoxide dismutase (SOD) were unexpected. Mn SOD is primarily a cytosolic enzyme, which is composed of four noncovalently associated identical subunits of approximately 22 kDa each, and which is principally found associated with the mitochondrial matrix in eukaryotes [41]. A bacterial cell wall SOD has been purified from *Nocardia asteroides* [42], whose unique localization of SOD may account, at least in part, for the ability of this pathogen to resist phagocytic attack. Furthermore, *N. asteroides* causes oxidative metabolic bursts in human polymorphonuclear neutrophils without itself being destroyed by the superoxides produced [43].

Osteoclasts, in addition to their role in normal bone turnover, are an active component of inflammatory responses, such as those occurring in rheumatoid arthritis and fracture repair [44]. It therefore seems possible that osteoclasts may have evolved cell surface protective mechanisms, including one analogous to that evolved by *N. asteroides*. Furthermore, the unusually large numbers of mitochondria in osteoclasts suggest that these cells experience a high level of metabolic activity. This O_2 metabolism results in the production of potentially harmful superoxides which must be removed in order to protect the cell from biological damage. Since a cell's polyanionic surface attracts hydrogen ions, creating a membrane microenvironment 2 to 3 pH units more acidic than the surrounding tissue fluid, the protonation of superoxide to form the even stronger oxidant, perhydroxyl, readily occurs at the cell surface. Moveover, transmembrane anion channels are capable of transporting superoxide radicals into the cell, consequently leading to intracellular molecular modifications which ultimately result in cell death [for a review of these effects, see 45]. The unique resorptive role of osteoclasts in bone biology may generate high levels of superoxides, either through direct cellular activity such as the maintenance of the acidic extracellular compartment, or as a byproduct of extracellular protein and matrix degradation. In the process of bone resorption, the osteoclast utilizes an anionic transporter to generate an acidic extracellular resorption lacuna [6]. The ability of these cells to readily create an acidic environment may result in unusually high conversion of superoxide to perhydroxyl radicals, unless there is a concomitant rapid removal of superoxide radicals, perhaps by an SOD-type activity at the plasma membrane. Furthermore, the high levels of anion transporters in the osteoclast plasma membrane may permit superoxide radicals present at the cell surface to readily move across the cell membrane, creating the potential for extensive intracellular damage. In this context, Webber et al.

[17] have demonstrated a correlation between expression of the antigen recognized by the 121F antibody and the resorptive activity of giant cells generated on the chick chorioallantoic membrane. This indirectly supports the hypothesis that the antigen may be, in some way, associated with the process of extracellular resorption.

The potential for contamination of our antigen preparation with an osteoclast mitochondrial SOD has not yet been unequivocably ruled out. However, two lines of evidence argue against this possibility. The mere 70% sequence homology observed between chicken liver mitochondrial SOD and the osteoclast antigen sequence virtually eliminates any potential for chicken mitochondrial SOD contamination. In addition, the substrate used to generate the hydroxylamine fragment was the 150 kDa region of an SDS-PAGE preparative gel, arguing strongly against a chance of contamination with a 22 kDa protein. We are presently exploring the use of HPLC purification to aid in obtaining additional amino acid sequence data.

In conclusion, we have identified and purified a high molecular weight osteoclast membrane glycoprotein using an osteoclast-specific monoclonal antibody. This protein can be characterized as an osteoclast-specific marker based on a number of tissue and cell specificity studies which indicate that the epitope recognized by the 121F monoclonal antibody is present on osteoclasts, but barely detectable or absent on other cells of the mononuclear phagocyte family [12,13,16,17]. Since this cell surface protein exists on mature osteoclasts, it seems unlikely that the protein is involved in promoting or establishing osteoclast differentiation. Marrow-derived multinucleated cells which develop either in the presence of live bone or live bone conditioned media express this antigen, whereas similar multinucleated cells which form in the absence of the bone environment do not [16]. Based on this recognition pattern, it seems more likely that this membrane component is involved in the activities of the mature phenotype. Results reported here and elsewhere [46] demonstrate both amino acid sequence homology and immunological cross-reactivity of the 150 kDa antigen with the Mn/Fe SOD family, and form the basis for a continuing line of investigation.

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